

Characterization of a New Metallo- β -Lactamase Gene, *bla*_{NDM-1}, and a Novel Erythromycin Esterase Gene Carried on a Unique Genetic Structure in *Klebsiella pneumoniae* Sequence Type 14 from India[▽]

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Received 10 June 2009/Returned for modification 7 August 2009/Accepted 10 September 2009

A Swedish patient of Indian origin traveled to New Delhi, India, and acquired a urinary tract infection caused by a carbapenem-resistant *Klebsiella pneumoniae* strain that typed to the sequence type 14 complex. The isolate, *Klebsiella pneumoniae* 05-506, was shown to possess a metallo- β -lactamase (MBL) but was negative for previously known MBL genes. Gene libraries and amplification of class 1 integrons revealed three resistance-conferring regions; the first contained *bla*_{CMY-4} flanked by *ISEcP1* and *btc*. The second region of 4.8 kb contained a complex class 1 integron with the gene cassettes *arr-2*, a new erythromycin esterase gene; *ereC*; *aadA1*; and *cmlA7*. An intact *ISCR1* element was shown to be downstream from the *qac/sul* genes. The third region consisted of a new MBL gene, designated *bla*_{NDM-1}, flanked on one side by *K. pneumoniae* DNA and a truncated *IS26* element on its other side. The last two regions lie adjacent to one another, and all three regions are found on a 180-kb region that is easily transferable to recipient strains and that confers resistance to all antibiotics except fluoroquinolones and colistin. NDM-1 shares very little identity with other MBLs, with the most similar MBLs being VIM-1/VIM-2, with which it has only 32.4% identity. As well as possessing unique residues near the active site, NDM-1 also has an additional insert between positions 162 and 166 not present in other MBLs. NDM-1 has a molecular mass of 28 kDa, is monomeric, and can hydrolyze all β -lactams except aztreonam. Compared to VIM-2, NDM-1 displays tighter binding to most cephalosporins, in particular, cefuroxime, cefotaxime, and cephalothin (cefalotin), and also to the penicillins. NDM-1 does not bind to the carbapenems as tightly as IMP-1 or VIM-2 and turns over the carbapenems at a rate similar to that of VIM-2. In addition to *K. pneumoniae* 05-506, *bla*_{NDM-1} was found on a 140-kb plasmid in an *Escherichia coli* strain isolated from the patient's feces, inferring the possibility of in vivo conjugation. The broad resistance carried on these plasmids is a further worrying development for India, which already has high levels of antibiotic resistance.

The growing increase in the rates of antibiotic resistance is a major cause for concern in both nonfermenting bacilli and isolates of the *Enterobacteriaceae* family. β -Lactams have been the mainstay of treatment for serious infections, and the most active of these are the carbapenems, which are advocated for use for the treatment of infections caused by extended-spectrum β -lactamase (ESBL)-producing *Enterobacteriaceae*, particularly *Escherichia coli* and *Klebsiella pneumoniae* (21). However, carbapenemases are increasingly being reported; and the most prevalent of these would appear to be KPC, which has recently been characterized in the United States, Israel, Turkey, China, India, the United Kingdom, and Nordic countries (20, 24, 26). KPC has invariably been found in *K. pneumoniae*, although recent reports indicate that it can cross species boundaries (2). OXA-48 is a class D carbapenemase that is

mainly found in *K. pneumoniae* and that is now reported from Turkey, China, India, and the United Kingdom (3, 5, 9). The mobile class B enzymes or metallo- β -lactamases (MBLs) have been mainly found in *Pseudomonas aeruginosa*, but they are increasingly being found in *Enterobacteriaceae*, particularly isolates from Greece and Turkey (39). Those mainly found in *K. pneumoniae* are the VIM-1/VIM-4 cluster (6).

The majority of resistance genes in *Enterobacteriaceae* are carried on class 1 integrons, and this is also true for the carbapenemases, such as VIM-1/VIM-4 (24, 40). In such cases, the genes are mobilized and evinced as gene cassettes and can theoretically move as separate entities independent of the integrase gene. Indeed, the majority of the mobile MBL genes are found as gene cassettes. These include *bla*_{IMP}, *bla*_{VIM}, *bla*_{GIM}, *bla*_{SIM}, and *bla*_{KMH} (41). The exception to these are genes encoding AIM-1 and SPM-1, which are adjacent to and thought to be mobilized by *ISCR* elements via a transposition event called rolling circle recombination (36).

Herein, we report on the genetic and biochemical characterization of a new subgroup of MBL, designated NDM-1, originating from New Delhi, India. We also report on its novel

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[▽] Published ahead of print on 21 September 2009.

genetic context and describe a new erythromycin resistance gene, designated *ereC*.

MATERIALS AND METHODS

Bacterial strains and susceptibility testing. The identification of *K. pneumoniae* 05-506 was performed with automated systems (the Phoenix and BD systems). Etest strips (AB bioMérieux, Solna, Sweden) were used to determine susceptibility, and the susceptibility results were interpreted according to the CLSI guidelines. *E. coli* J53 and *E. coli* TOPO (Stratagene, Amsterdam, The Netherlands) were used in the conjugation and cloning experiments, respectively.

Phenotypic and molecular detection of MBL. Hodge and imipenem-EDTA double-disc synergy tests as well as Etest were used to screen for MBL production. In addition, the carbapenemase activities of cell sonicates from overnight broth cultures (Mueller-Hinton broth; BD) were determined by spectrophotometric assays. These were undertaken by using 150 μ M imipenem as the substrate and 299 nm for the measurement of hydrolysis. The assays were performed with or without EDTA (25 mM) to examine the inhibition of carbapenemase activity. The strains were screened for the presence of known mobile MBL genes (*bla*_{VIM}, *bla*_{IMP}, *bla*_{SPM-1}, *bla*_{GIM-1}, *bla*_{SIM-1}, *bla*_{AIM-1}) by PCR with the primers reported previously (22). The strains were also screened for the presence of other β -lactamase genes (*bla*_{CTX}, *bla*_{CMY}, etc.) as well as class 1, 2, and 3 integron structures (24). Strains known to harbor MBL genes were used as positive controls for the PCR analysis.

Conjugation. *E. coli* J53 was used as the recipient in the conjugation studies, and mating was carried out on blood agar medium without selection (25). After 18 h, the mixed cultures were taken from the plates, suspended in saline, and plated onto MacConkey medium containing sodium azide (100 μ g/ml) and ceftazidime (8 μ g/ml). Confirmation that conjugation had taken place in *E. coli* J53 was carried out by indole testing and testing for the presence of the MBL gene by PCR analysis.

DNA cloning. Cloning experiments were performed by using the cloning vector pK18, as described previously (35). Restriction endonucleases BamHI, EcoRI, and HindIII (Invitrogen, Carlsbad, CA) were used to cut both plasmid and *K. pneumoniae* 05-506 chromosomal DNA. Transformation was carried out by using electroporation and *E. coli* TOP10 cells (Invitrogen Ltd, Paisley, United Kingdom). Transformants were selected on LB agar plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (30 μ g/ml), ceftazidime (6 μ g/ml), and kanamycin (25 μ g/ml). Recombinant plasmids were recovered with a QIAprep spin miniprep kit (Qiagen, West Sussex, United Kingdom). Restriction endonuclease analysis of the plasmid was carried out with a variety of restriction enzymes to determine the size of the ligated nucleotide segment.

The PCR-amplified open reading frame of NDM-1 was digested with the appropriate enzymes and was subsequently ligated into the pVFT3S (six-His—*trx* fusion vector) bacterial expression vector. The construct was transformed into *E. coli* BL21 *groEL/groES* (pGro7/BL21; Takara Bio Inc., Japan).

DNA sequencing and computer analysis. Both strands of the clones from the libraries that were produced were sequenced by using M13 forward and M13 reverse primers. The cloned insets containing *bla*_{NDM-1}, the complex class 1 integron, and *bla*_{CMY-4} were fully sequenced. The nucleotide sequences, deduced amino acid sequences, and phylogenetic relationships were analyzed by using the Lasergene software package (DNASTar, Madison, WI). The sequences obtained were compared to the sequences available on the Internet (<http://www.ncbi.nlm.nih.gov/blast/>).

Pulsed-field gel electrophoresis (PFGE) and genomic DNA digestion with endonucleases. Genomic DNA was prepared in agarose blocks and digested with the restriction enzymes XbaI and SpeI (Roche Diagnostics, Mannheim, Germany), I-CeuI (New England Biolabs, Beverly, MA), and S1 nuclease (Invitrogen, Abingdon, United Kingdom). The DNA fragments were separated by use of a CHEF-DR III apparatus (Bio-Rad, Hercules, CA) for 20 h at 6 V/cm and 14°C with initial and final pulse times of 0.5 and 30 s, respectively.

Southern blot analysis. Southern blotting was performed on agarose gels by in-gel hybridization with the *bla*_{NDM-1} probe labeled with ³²P (Stratagene) and by a recently described random primer method (22).

MLST. Multilocus sequence typing (MLST) of *K. pneumoniae* was performed as described by Diancourt et al. (7). Experimentally determined DNA sequences were uploaded into the MLST database (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html>), and allelic numbers as well as sequence types (STs) were obtained.

β -Lactamase purification. Cell lysates were centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was obtained and loaded onto Ni-nitrilotriacetic acid agarose. The NDM-1 protein was eluted by the use of elution buffer with 300

mM imidazole. The protein was separated from the His-*trx* tag by tobacco etch virus protease and was finally purified by gel filtration on a GS320 column.

Kinetic measurements. Purified β -lactamase was used to determine the kinetic parameters k_{cat} and K_m . Reactions were performed at 30°C in 1 ml of assay buffer (10 mM HEPES, pH 7.5). The assays were performed in a UV-1601PC spectrophotometer (Shimadzu Corp., Tokyo, Japan) by observing the changes in absorption resulting from the opening of the β -lactam ring at the specific wavelengths for each of the antimicrobial agents evaluated, as described previously (18).

Nucleotide sequence accession number. The nucleotide sequence data reported in the present study have been assigned EMBL/GenBank nucleotide accession number FN396876.

RESULTS

Clinical details for *K. pneumoniae* 05-506. A 59-year-old male patient who was originally from India but who had lived in Sweden for many years and who often returned to India had type 2 diabetes mellitus and had had multiple strokes. In November 2007, he traveled to India and on 5 December was hospitalized in Ludhiana, Punjab, with a large gluteal abscess. In December 2007, he was admitted to a hospital in New Delhi, where he was again operated on and where he developed a decubital ulcer. On 8 January 2008 he was referred to Örebro, Sweden. During his stay in New Delhi he received amoxicillin (amoxicilline)-clavulanic acid, metronidazole, amikacin, and gatifloxacin (all of them parenterally).

Clinical isolate *K. pneumoniae* 05-506 was derived from a urinary culture on 9 January 2008. He had no clear symptoms of urinary tract infection at that time. The amount of bacteria found in a culture of his urine was only 1,000 CFU/ml. Both ESBL-positive *Escherichia coli* and a carbapenem-susceptible *Acinetobacter* sp. were isolated from his deep wounds. An ESBL-positive *E. coli* strain was also found in a culture of fluid resulting from external otitis.

On 6 March 2008, the patient was discharged to a nursing home. On 1 April a new urine sample for culture was taken, and an ESBL-producing *K. pneumoniae* isolate was found. The original carbapenem-resistant *K. pneumoniae* isolate has never been found in any other cultures of samples from the patient. As *K. pneumoniae* 05-506 was carbapenem resistant and positive by the MBL Etest (AB bioMérieux), it was investigated further. Moreover, fecal samples were collected from the patient during his stay at the nursing home to identify the source of 05-506; however, while 05-506 could not be recovered, an MBL-positive *E. coli* isolate was recovered and was designated *E. coli* NF-NDM-1.

Typing and phenotypic characterization of *K. pneumoniae* 05-560, *E. coli* NF-NDM-1, and transformants. ST14 is a single-locus variant of ST15, which has been encountered with KPC- and CTX-M-producing *K. pneumoniae* strains (19, 30). We have also found ST14 among U.S. KPC producers (19).

The susceptibility patterns of *K. pneumoniae* 05-560, *E. coli* NF-NDM-1, *E. coli* TOP10, *E. coli* TOP10(pNDM-1), *E. coli* J53, and *E. coli* J53(pNDM-1) are listed in Table 1. *K. pneumoniae* 05-560 was resistant to all β -lactams and was sensitive only to colistin (Table 1). The susceptibility profile of *E. coli* NF-NDM-1 largely reflects that of 05-560, apart from susceptibility to aztreonam, cefepime, and ciprofloxacin, to which the level of resistance of 05-560 was not quite as high. When the cloned MBL gene and its adjacent DNA were expressed in an *E. coli* background, the strain was highly resistant to all cephe-

TABLE 1. Antimicrobial susceptibility patterns of *K. pneumoniae* 05-560, *E. coli* NF-NDM-1, transformants, and conjugants

Antibiotic	MIC ($\mu\text{g/ml}$)					
	<i>K. pneumoniae</i> 05-560	<i>E. coli</i> TOP10	<i>E. coli</i> TOP10(NDM-1)	<i>E. coli</i> J53	<i>E. coli</i> J53(pNDM-1)	<i>E. coli</i> NF-NDM-1
Ampicillin	>256	12	>256	4	>256	>256
Piperacillin	>256	0.5	NT	1	>256	>256
Cephalothin	>256	8	>256	4	>256	>256
Cefoxitin	>256	4	>256	2	>256	>256
Cefotaxime	>256	0.125	>256	0.064	>256	>256
Cefuroxime	>256	8	>256	8	>256	>256
Ceftazidime	>256	1	>256	0.125	>256	>256
Aztreonam	>256	0.094	0.25	0.064	24	24
Cefepime	>256	0.032	8	0.064	24	24
Ertapenem	>32	0.125	24	0.5	>32	>32
Imipenem	>32	0.094	12	0.25	16	>32
Meropenem	>32	0.064	12	0.064	>32	>32
Ciprofloxacin	>32	0.004	0.006	0.032	0.047	4
Colistin	0.75	0.38	0.38	0.25	0.25	0.25

alosporins, apart from cefepime (MIC, 8 $\mu\text{g/ml}$), and was sensitive to aztreonam. However, the aztreonam MICs differed significantly for TOP10 *bla*_{NDM-1} (MIC, 0.25 $\mu\text{g/ml}$) and J53 *bla*_{NDM-1} (MIC, 24 $\mu\text{g/ml}$), even though the aztreonam MICs for TOP10 and J53 were very similar. Interestingly, the MICs of imipenem and meropenem were relatively high for both *E. coli* TOP10 and J53 compared with those for strains with the other cloned MBL genes (31, 41).

Phenotypic and molecular screening for MBLs. The results of the MBL screening tests were positive by the imipenem-EDTA double-disc synergy test, the MBL Etest (256 $\mu\text{g/ml}$ with imipenem and 3 $\mu\text{g/ml}$ with imipenem plus inhibitor), and the determination of carbapenemase activities by spectrophotometry (data not shown). However, PCR analysis failed to detect previously known MBL genes when the control genes *bla*_{VIM}, *bla*_{IMP}, *bla*_{SPM-1}, *bla*_{GIM-1}, *bla*_{SIM-1}, *bla*_{AIM-1} were used. PCR did detect a class 1 integron (*intI* and *qacE Δ 1/sul*) of 4.8 kb.

Nucleotide and deduced amino acid sequences of three resistance-conferring regions. Total DNA from *K. pneumoniae* 05-560 was used to construct a genomic library to isolate all genes conferring β -lactam resistance. After the plasmids of resistant colonies were analyzed by restriction profiling, it was evident that there were two different types of β -lactam resistance. One group carried a putative AmpC enzyme, on the basis of the fact that it was resistant to the effects of EDTA but sensitive to inhibition with cloxacillin. The other group possessed a putative MBL on the basis of the fact that it was resistant to the effects of cloxacillin and clavulanic acid but sensitive to EDTA. Restriction of the recombinant plasmids showed that the AmpC-type genes are carried on a 2.1-kb fragment and that the MBL gene is carried on a 4.3-kb fragment. Additionally, the 4.8-kb fragment containing the class 1 integron was also sequenced.

The class 1 integron contains a unique set of genes, some of which have been previously reported from Asia. Immediately next to the *intI* gene is an *arr-2* cassette that mediates resistance to rifampin (rifampicin) and that has been found in isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* from China and Hong Kong, respectively (GenBank accession numbers EU886979 and AY038837, respectively) (1, 14,

27, 38). It has also been identified on the chromosome of *Streptomyces coelicolor* (GenBank accession number NC003888). Next to *arr-2* is a novel erythromycin esterase gene (*ereC*) which has 92% nucleotide sequence identity with *ereA* from *E. coli* (GenBank accession number AY183453) (Fig. 1). The predicted protein sequence differs from the EreA sequence at an additional 10 amino acids at the N terminus and also has 31 amino acid substitutions throughout the sequence. At present, only two erythromycin esterases, *ereA* and *ereB*, have been found (GenBank accession numbers DQ157752 and X03988, respectively), and these share only 25% identity in their amino acid sequences (Fig. 1). Adjacent to the esterase gene is *aadA1*, which mediates resistance to gentamicin, and the *cmlA7* gene cassette, which mediates resistance to chloramphenicol, is present between *aadA1* and *qac/sul* (Fig. 2).

The AmpC fragment is shown in Fig. 2 and carries *bla*_{CMY-4}, which is responsible for the AmpC phenotype. Upstream of *bla*_{CMY-4} is an intact copy of *ISEcP1*, which is probably responsible for the transposition and mobility of *bla*_{CMY-4}. Downstream is *blc*, which is a common feature in many *bla*_{CMY} regions and which has been reported by Kang et al. (16).

The 4.3-kb fragment contained a novel MBL gene, designated *bla*_{NDM-1} (Fig. 2). This section of DNA was a chimera of truncated and intact genes. The *bla*_{NDM-1} gene was found between a section of *K. pneumoniae* chromosomal DNA and truncated sections of the IS26 and Tn3 transposase genes. The *Klebsiella* DNA consisted of a putative efflux protein gene adjacent to another gene encoding L-lactate hydrogenase, and both were 99.5% identical to a section of chromosomal DNA from *K. pneumoniae* MGH78578 (GenBank accession number CP000647) and found in the same orientation in which they are present on the *Klebsiella* chromosome. The lactate dehydrogenase gene was truncated and missing approximately 750 bp of the 5' end of the gene. Immediately upstream of the lactate dehydrogenase gene was a short internal part of the *ampC* gene, *bla*_{DHA-1} (base pair coordinates 1268 to 1478), consisting of the DNA encoding amino acids 196 to 265. This gene was truncated at both ends both by the lactate dehydrogenase gene and by a section of DNA encoding the 5' end of a phosphoribosyl anthranilate isomerase gene. This section of DNA (coordinates 1478 to 2100) has identities ranging from 55% to 68%



FIG. 1. Alignment of novel erythromycin esterase EreC (this study) with EreA and EreA2. Amino acid changes between EreC and EreA are indicated with asterisks. The single amino acid difference between EreA and EreA2 is highlighted in gray.

to the same gene from various members of the alphaproteobacteria, including *Rhodobacter* spp., *Rhodospseudomonas* spp., *Caulobacter* spp., and *Novosphingobium* spp. The highest degree of identity was found with *Novosphingobium aromaticivor*, with 68% nucleotide sequence identity over 575 nucleotides. Again, this gene was truncated and missing the DNA encoding the last 19 amino acids of the C terminus of the protein. The bla_{NDM-1} gene is encoded within a 1,350-bp section of DNA with a lower G+C content (57%) than that of the surrounding DNA, which has a G+C content of 62 to 65% and which consists of a gene of 807 nucleotides and flanking DNA on either side. Upstream of the bla_{NDM-1} gene is 250 nucleotides of DNA that contains several sequences that show similarity to conventional promoter sequences, and it is likely that

the promoter driving the expression of bla_{NDM-1} has been acquired along with the gene (Fig. 2). Farther upstream is a truncated left-hand end of an IS26 insertion sequence, including 270 bp of the left-hand end of the element with the left-hand inverted repeat and DNA encoding the first 74 amino acids of the transposase. This element lies adjacent to a *tnpA* gene of a Tn3-like transposon (coordinates 3748 to 4318), which is itself truncated and missing the 5' end of the gene encoding the first 128 amino acids of the transposase (Fig. 2).

Structural properties of NDM-1. The bla_{NDM-1} open reading frame encodes a putative protein of 269 amino acids with a molecular mass of approximately 27.5 kDa (Fig. 3). The NDM-1 sequence and the behavior of NDM-1 during gel filtration and mass spectrometry (data not shown) indicate that it

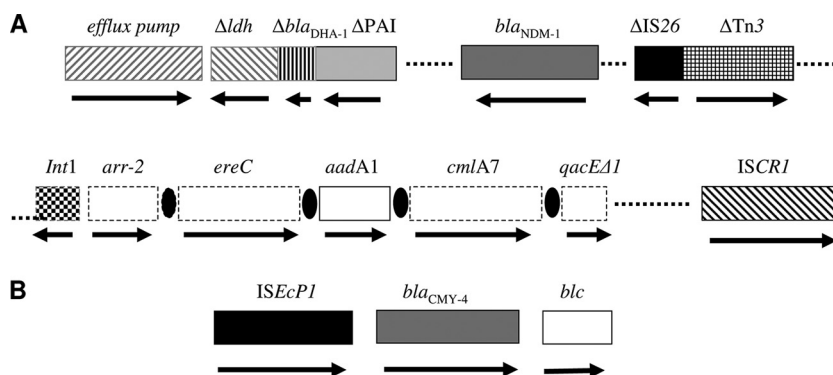


FIG. 2. Three characterized antibiotic resistance-conferring regions from *K. pneumoniae* 05-506. (A) The 4.3-kb region is linked to the 4.8-kb complex class 1 integron region. The genes encoding the efflux pump and lactate dehydrogenase (gray diagonal lines) are of *Klebsiella* origin. bla_{NDM-1} (dark gray) is flanked between the pathogenicity island (vertical black lines) and IS26/Tn3 (black small squares). This region lies downstream of the 4.8-kb complex class 1 integron containing *Int* (checkered area), *arr-2*, *ere2A*, *aadA1*, and *cmlA7* as gene cassettes and *qacA1* (white boxes). Downstream is an intact copy of *ISCR1* (black and white diagonal lines). Arrows, direction of transcription; black ellipses, 59-base elements; Δ, genes that are truncated. (B) bla_{CMY-4} (gray) is located between ISEcP1 (black) and *blc* (white), as reported previously (16).

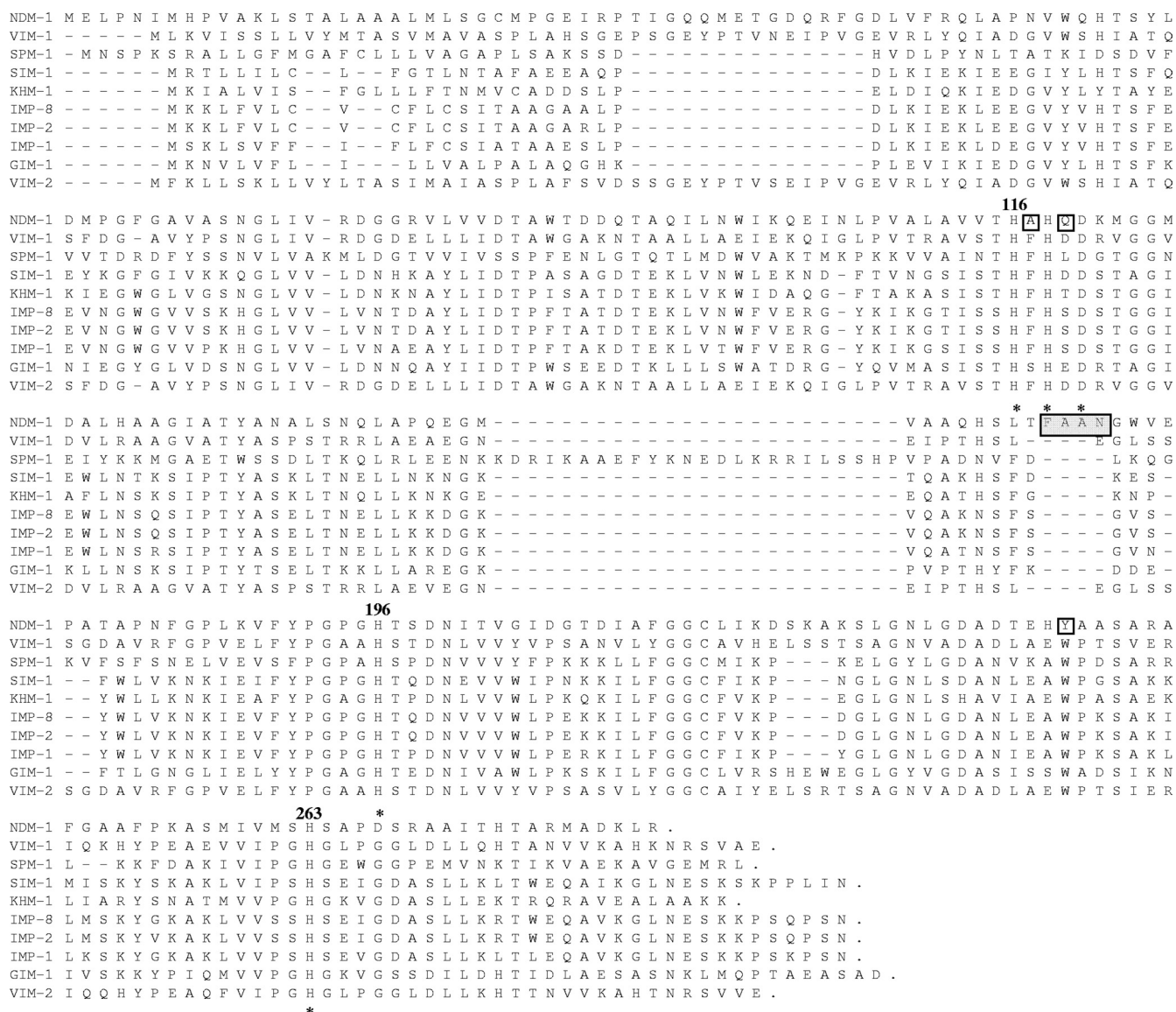


FIG. 3. Alignment of the amino acid sequences of NDM-1 with the amino acid sequences of IMP-1, IMP-2, IMP-8, VIM-1, VIM-2, GIM-1, SPM-1, SIM-1, and KHM-1. Conserved residues coordinating zinc ions are denoted with asterisks. Key amino acids are also numbered according to the BBL scheme (12). Additional amino acids unique to NDM-1 are enclosed in a light gray box. Amino acid substitutions near the active site are highlighted in plain boxes (13).

is actively present as a monomer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed an approximate molecular mass of 28 kDa (data not shown). NDM-1 possesses a leader peptide, with the probable cleavage site occurring at position 19 between two alanines which are in a very similar position to the cleavage site for the VIM MBLs (Fig. 3). NDM-1 also shares the additional loop of the VIM enzymes at positions 34 and 47 but also possesses a unique additional sequence at positions 162 to 166 (*bla*_{NDM-1} and not BBL numbering) (Fig. 3) (4). NDM-1 shares very little identity with other MBLs and is the most closely related to VIM-1/VIM-2, with which it has only 32.4% identity (Fig. 4). Curiously, NDM-1 also has a unique HXHXD motif among the mobile MBLs, as it contains an alanine between the two histidines. NDM-1 also possesses a tyrosine at position 222 in

stead of the universally conserved tryptophan (Fig. 3). The mature peptide has a theoretical pI of 6.9.

Functional properties of NDM-1. Analysis of the purified NDM-1 enzyme by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed a single band corresponding to a size of 28 kDa (data not shown). Table 2 lists the k_{cat} and K_m values and compares them with the kinetic values of IMP-1 and VIM-2. NDM-1 shows tight binding (low K_m values) to most cephalosporins and, in particular, to cefuroxime, cefotaxime, and cephalothin (cefalotin). It also shows relatively tight binding to the penicillins, which is unusual for an MBL, as both IMP-1 and VIM-2 possess higher K_m values (Table 2). Interestingly, NDM-1 does not bind to the carbapenems as tightly as IMP-1 or VIM-2. The rate of turnover (k_{cat}) of penicillins by NDM-1 is lower than that by IMP-1 and VIM-2 and is gener-

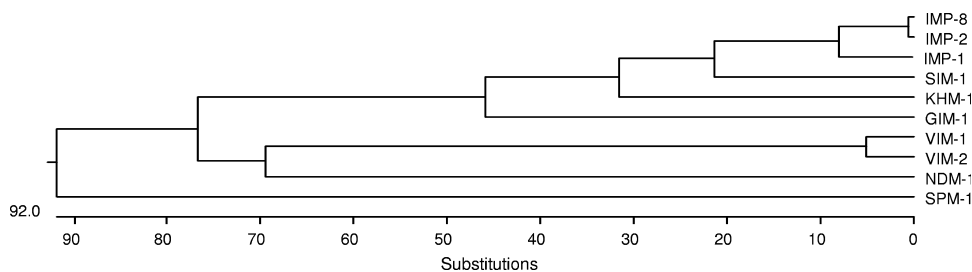


FIG. 4. Phylogenetic tree obtained for MBL subgroup B1. The numbers for the substitutions should be multiplied by 100. The alignment used was prepared with the CLUSTALW program. The GenBank accession numbers for the sequences are as follows: IMP-1, S71932; IMP-2, AJ243491; IMP-8, AF322577; VIM-1, Y18050; VIM-2, AF191564; GIM-1, AJ620678; SPM-1, AJ492820; SIM-1, AY887066; KHM-1, AB364006.

ally unusual for MBLs. The k_{cat} values for the cephalosporins are more similar to those of IMP-1 than to those of VIM-2, yet the k_{cat} values for the carbapenems (20 and 12 s⁻¹ for imipenem and meropenem, respectively) are more similar to those for VIM-2 (10 and 1 s⁻¹, respectively) than IMP-1 (46 and 50 s⁻¹, respectively) (Table 2). Due to the fact that most of the K_m values are low, the k_{cat}/K_m ratios are reasonably high and comparable to those of VIM-2. The exception is that of ceftazidime, which has a K_m of 181 μM and a k_{cat} of 5 s⁻¹ and which thus presents with a k_{cat}/K_m of 0.03.

Plasmid analysis and back probing with *bla*_{NDM-1}. The plasmids carrying *bla*_{NDM-1} from both *K. pneumoniae* 05-506 and *E. coli* NF-NDM-1 were successfully transferred to *E. coli* J53 at a frequency of approximately 10⁻⁴ to 10⁻⁵. PCR was undertaken with primers directed to *ISCR1* and *bla*_{NDM-1}, and both were shown to be carried by pNDM-1. To examine the sizes of the plasmids from 05-506, *E. coli* J53(pNDM-1), and *E. coli* NF-NDM-1, genomic DNA was isolated from each of the strains, restricted with S1 nuclease, and examined by PFGE. The corresponding gel was blotted with *bla*_{NDM-1}, and the data clearly show that *bla*_{NDM-1} is on a 180-kb plasmid in 05-506 and *E. coli* J53(pNDM-1), but in *E. coli* NF-NDM-1 it is on a smaller plasmid of 140 kb (Fig. 5). Inc typing was unsuccessful, and plasmid pNDM-1 is currently being sequenced to identify

the origin of replication and other functions carried by pNDM-1.

As the broader genetic context was not known and *ISCR1* had been found in a large antibiotic resistance-conferring region, *K. pneumoniae* 05-506 was also digested with XbaI, SpeI, I-CeuI, and S1 nuclease and was then probed with radiolabeled *bla*_{NDM-1}. The probing results were compared to those of probing of *ISCR1* to ascertain whether *bla*_{NDM-1} and *ISCR1* are on the same genetic structure (Fig. 6). The probing data for *ISCR1* and *bla*_{NDM-1} are identical and clearly indicated probing to the same 25-kb region, suggesting that the regions are adjacent to one another.

DISCUSSION

K. pneumoniae 05-506 types as ST14, a single-locus variant of type ST15. Recently, *K. pneumoniae* ST15 has been branded as a new methicillin-resistant *S. aureus* type of pathogen due to its wide distribution and its carriage of *bla*_{CTX-M15} and other resistance markers (19, 30). Strain 05-506 clearly arose in India, but there are few data arising from India to suggest how widespread it is. We are currently undertaking studies in several Indian cities to examine these points. Interestingly, there appears to be the possible transfer of *bla*_{NDM-1} in vivo either

TABLE 2. Steady-state kinetic constants of NDM-1, IMP-1, and VIM-2

Compound	NDM-1			IMP-1 ^a			VIM-2 ^b		
	K_m (μM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ / μM)	K_m (μM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ / μM)	K_m (μM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ / μM)
Penicillin G	16	11	0.68	520	320	0.62	49	56	1.14
Ampicillin	22	15	0.66	200	950	4.8	DNA		
Piperacillin	12	14	1.17	ND ^c	ND	ND	72	33	0.45
Cephalothin	10	4	0.40	21	48	2.4	44	57	1.28
Cefoxitin	49	1	0.02	8	16	2	24	3	0.12
Cefotaxime	10	6	0.58	4	1.3	0.35	32	28	0.86
Cefuroxime	8	5	0.61	37	8	0.22	22	12	0.55
Ceftazidime	181	5	0.03	44	8	0.18	98	89	0.90
Aztreonam	ND			>1,000	>0.01	<0.0001	ND	<0.5	ND
Cefepime	77	13	0.17	11	7	0.66	184	5	0.03
Imipenem	94	20	0.21	39	46	1.2	10	10	0.99
Meropenem	49	12	0.25	10	50	0.12	5	1	0.28
Clavulanic acid	ND			NR ^d			NR		

^a From Spencer et al. (32).

^b From Poirer et al. (23).

^c ND, not detected.

^d NR, not reported.

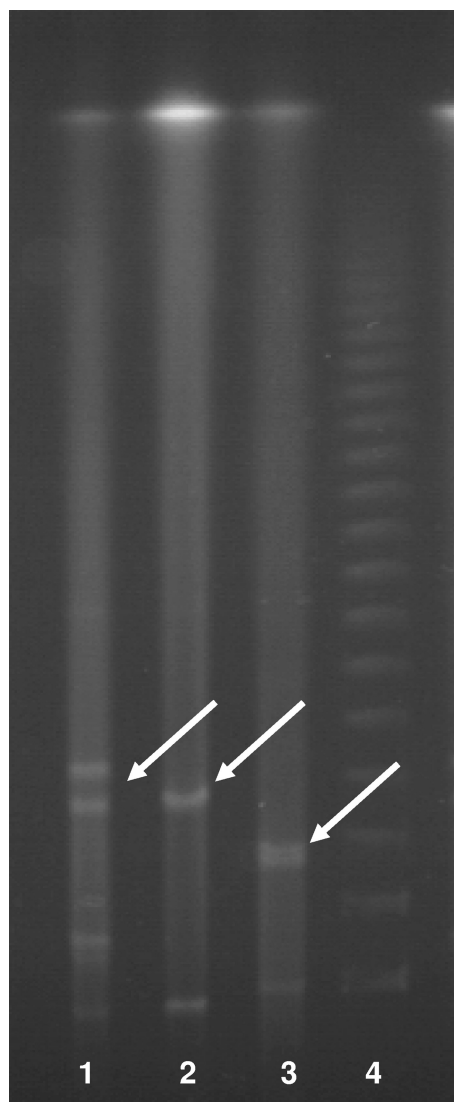


FIG. 5. PFGE of plasmids from *K. pneumoniae* 05-506, conjugant *E. coli* J53(pNDM-1), and *E. coli* NF-NDM-1 (from the patient's normal flora). The bands with white arrows showed positive signals by Southern blot hybridization with the NDM-1 probe (data not shown). *K. pneumoniae* 05-506 carries *bla*_{NDM-1} on a 180-kb plasmid, whereas *E. coli* NF-NDM-1 carries *bla*_{NDM-1} on a plasmid of approximately 140 kb.

from *K. pneumoniae* to *E. coli* or vice versa, but more interestingly, the plasmids carrying *bla*_{NDM-1} in the two species are of different sizes. This evidence would suggest that there is rearrangement in vivo which could result from either duplication and insertion, e.g., transposition or rolling circle replication from the smaller plasmid, or deletion from the larger plasmid (33, 34). The plasmid carrying *bla*_{NDM-1} also carries *bla*_{CMY-4} and the complex class 1 integron carrying several antibiotic resistance-conferring genes (33), and it has also shown itself to naturally have a broad host range. When the plasmid was transferred to *E. coli* J53, the *E. coli* strain containing pNDM-1 was resistant to all antibiotics except colistin and ciprofloxacin and was shown by blotting and PCR to carry *bla*_{CMY-4}, the ISCR region, and *bla*_{NDM-1}. Therefore, the rapid

dissemination of this plasmid among clinical bacteria would be a nightmare scenario.

This is the first report of an MBL gene not carried in a class 1 integron or adjacent to ISCR1 elements. It appears to have an intact promoter sequence that is not dependent on the IS26 element inserted upstream, even though IS26 is found adjacent to other resistance genes and pathogenicity islands (8, 10, 11, 15). The plasmid clearly contains *Klebsiella* housekeeping genes (Fig. 2) as part of this capturing DNA machinery, and therefore, these have probably been purloined from the *Klebsiella* chromosome. Furthermore, restriction digests and probing experiments indicate that *bla*_{NDM-1} and ISCR1 are on a DNA fragment of less than 25 kb (Fig. 6). While Tn3 and IS26 are capable of movement, the amelioration of such a large region spanning from *bla*_{NDM-1} to ISCR1 could have occurred only through a series of duplications and homologous recombination (34). The known location of ISCR1 in all sequences analyzed so far is at the end of a complex class 1 integron that is more often than not contained within a Tn3-like transposon, and therefore, it is possible that the ISCR1 element is upstream of the *bla*_{NDM-1} gene and farther downstream of the Tn3 transposase. Additionally, the chimeric structure of the 4.3-kb insert with its composition of fused truncated and nontruncated genes is a classic signature of ISCR1 transposition activity (22, 34, 37).

NDM-1 not only is a new subclass of the B1 group of MBLs but also possesses novel amino acids near the active site, suggesting that it has a novel structure. NDM-1 possesses only 32.4% identity with VIM-2, and therefore, molecular replacement or threading cannot be undertaken to reasonably ascertain its structure. However, NDM-1 does show some similarities to the VIM group of MBLs; namely, it possesses the additional sequences at positions 226 to 228 (BBL numbering) not present in other MBLs (12). These residues contain R228 in VIM-2, which is thought to stabilize C221 and possibly H263, yet in NDM-1 the substitute R228A is unlikely to play such a role. VIM-2 also possesses Y224, which is in close proximity to C221, whereas NDM-1 has a lysine, which is likely to serve a similar role. Crystallographic insights into the mechanism of action of VIM-2 by Garcia-Saez et al. suggest that Y67 pivots in toward the enzyme away from bulk solvent and in so doing stabilizes H263; but NDM-1, like other non-VIM MBLs, possesses the substitution Y67V, which is unlikely to function in the same manner (13). However, NDM-1 does possess S69, which is in close proximity to Y67 and less so to R121 and H263 (13). The role of these substitutions is not apparent, as NDM-1 possesses kinetics similar to those of VIM-2 (23, 29). In general, NDM-1 binds more tightly (it has a lower K_m) to most β -lactam substrates and hydrolyzes them less well (it has a lower k_{cat}), with the exception being the less bulky carbapenems, for which NDM-1 possesses relatively high K_m and k_{cat} values for both imipenem and meropenem (23, 29).

In addition to *bla*_{NDM-1}, this study also characterized a new erythromycin esterase gene, designated *ereC* (28). The esterase gene is part of the complex class 1 integron containing the Asian rifampin resistance gene, *arr-2*, which is also contained as a gene cassette (17). If this structure is stable, together with *bla*_{NDM-1}, it will act as a unique genetic marker for the prevalence and stability of plasmid pNDM-1 throughout Indian

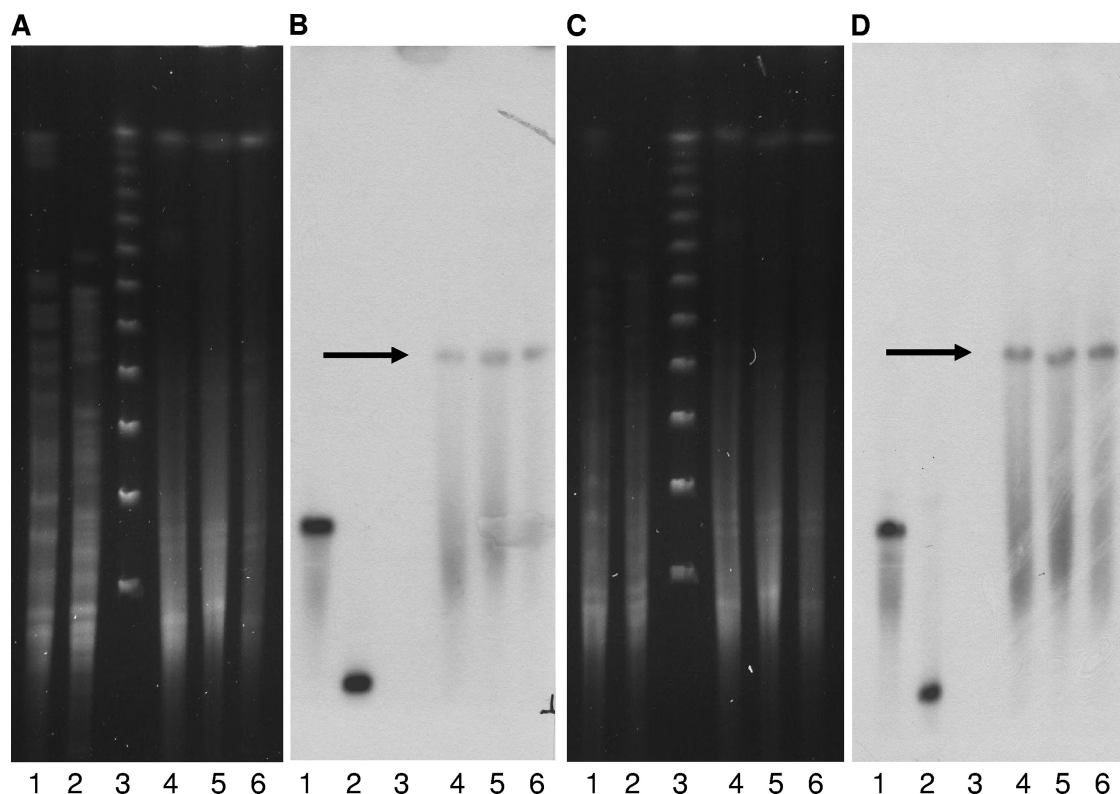


FIG. 6. PFGE findings (A and C) and Southern blot hybridization (B and D) with *bla*_{NDM-1} (A and B) and an ISCRI probe (C and D). The genomic DNA of the index strain (*K. pneumoniae* 05-506) was undigested (lane 1) or was digested with S1 nuclease (lane 2), XbaI (lane 4), SpeI (lane 5), or I-CeuI (lane 6). Lane 3, DNA size marker.

Enterobacteriaceae strains. We are currently doing molecular epidemiological studies of Indian *Enterobacteriaceae* carbapenem-resistant strains to examine the prevalence and range of pNDM-1 among clinical and nonclinical isolates. In a country where there is little control on antibiotic prescriptions, the rapid dissemination of such a plasmid is alarming.

ACKNOWLEDGMENTS

This work was funded by EU grant LSHM-CT-2005-018705 and Wellcome Trust grant 084627/Z/08.

D. Yong thanks Seok H. Jeong and Yunsop Chong for their valuable advice.

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